

Research Article

# Multiplex 16S rRNA-derived geno-biochip for detection of 16 bacterial pathogens from contaminated foods

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Foodborne diseases caused by various pathogenic bacteria occur worldwide. To prevent foodborne diseases and minimize their impacts, it is important to inspect contaminated foods and specifically detect many types of pathogenic bacteria. Several DNA oligonucleotide biochips based on 16S rRNA have been investigated to detect bacteria; however, a mode of detection that can be used to detect diverse pathogenic strains and to examine the safety of food matrixes is still needed. In the present work, a 16S rRNA gene-derived geno-biochip detection system was developed after screening DNA oligonucleotide specific capture probes, and it was validated for multiple detection of 16 pathogenic strains that frequently occur with a signature pattern. rRNAs were also used as detection targets directly obtained from cell lysates without any purification and amplification steps in the bacterial cells separated from eight food matrixes by simple pretreatments. Thus, the developed 16S rRNA-derived geno-biochip can be successfully used for the rapid and multiple detection of the 16 pathogenic bacteria frequently isolated from contaminated foods that are important for food safety.

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## 1 Introduction

Foodborne diseases occur worldwide. Approximately 300 cases of outbreaks involving more than 6000 patients annually are reported in Korea [1, 2]. Mild symptoms include diarrhea, nausea, vomiting, and fever [3]. However, severe symptoms, such as sepsis, hemolytic uremic syndrome, and/or death, sometimes arise in certain patients, such as infants, older adults, or immunocompromised patients [4, 5]. Foodborne diseases are mainly caused by various pathogenic bacteria, including *Campylobacter* spp., *Shigella* spp., *Salmonella* spp., and pathogenic *Escherichia coli* [3, 5]. Therefore, to prevent foodborne diseases and minimize the impact of foodborne

outbreaks caused by contaminated foods and agricultural products, it is important to inspect contaminated foods and to detect diverse pathogens accurately and simultaneously.

For this reason, a large number of detection and diagnosis methods have been developed. Culture-based conventional methods are commonly used to detect bacteria, but they are time-consuming, labor-intensive, and difficult to quantitatively analyze [6, 7]. Therefore, diverse rapid technologies have been devised from molecular-based methods such as polymerase chain reaction (PCR) [8–12], in situ hybridization assays [13, 14], and microarrays [15–19]. In particular, the DNA microarray is considered to be a potent device that allows multiple, specific, and sensitive screening for pathogen identification. DNA oligonucleotide probes with a short length (≈20–80 bp) in arrays can improve the accuracy and reproducibility of results by increasing specificity and standardizing the hybridization process compared to longer DNA probes

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**Abbreviations:** PCR, polymerase chain reaction; RDP, Ribosomal Database Project

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[20]. Several DNA oligonucleotide biochips based on 16S rRNA information have been investigated to detect bacteria, and 16S rRNA containing conserved and variable sequences has been widely used for phylogenetic discrimination [4, 21–23]. Previously, we developed the 16S rRNA-based microarrays for detection of seven pathogens from pure culture [16] and 11 pathogens with pattern-mapping [18]. In addition, the detection technique was developed with 16S rRNA targets [19]. However, a detection method that can be used to identify more diverse pathogenic strains and can be used to check the safety of food matrixes is still necessary.

In the present work, a 16S rRNA-derived geno-biochip system was developed for the multiple detection of 16 pathogenic bacteria (*Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serotype Choleraesuis, *Salmonella enterica* subsp. *enterica* serotype Enteritidis, *Shigella boydii*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Yersinia enterocolitica*), which frequently occurred worldwide in agricultural products [1, 24], using rRNAs as detection targets directly from cell lysates without any purification and/or amplification process. In addition, a simple pretreatment method was employed to separate bacterial cells from various food matrixes.

## 2 Materials and methods

### 2.1 Pathogenic strains

For the design of specific capture probes, 23 strains of 16 microbial species were used (Supporting information, Table S1): *B. cereus* (American Type Culture Collection (ATCC; Manassas, VA, USA) 11778, 13061, and 14579), *C. jejuni* (ATCC 33291), *C. perfringens* (ATCC 13124), *E. coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 43894), *L. monocytogenes* (ATCC 15313), *S. boydii* (ATCC 8700 and 35966), *S. dysenteriae* (ATCC 13313), *S. sonnei* (ATCC 9290, 25931, and 29930), *S. aureus* (ATCC 6538), *S. enterica* subsp. *enterica* serotype Choleraesuis (ATCC 7001, 10708, and 13312), *S. enterica* subsp. *enterica* serotype Enteritidis (ATCC 31194), *V. cholerae* (ATCC 14035), *V. parahaemolyticus* (ATCC 17802), *V. vulnificus* (ATCC 27562), and *Y. enterocolitica* (ATCC 23751).

### 2.2 Design and synthesis of DNA oligonucleotide specific capture probes

16S rRNA sequences of all bacterial strains were acquired from the Ribosomal Database Project (RDP) II database (Centers for Microbial Ecology, East Lansing, MI, USA) or the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/gene>) Genbank. Their

sequences were also directly obtained by sequencing polymerase chain reaction (PCR) amplicons to compare them with those from databases (data not shown). Because 16S rRNA sequences show high similarity between closely related species, the closest comparison method was employed, that is, species-specific regions were checked by the alignment of the most similar two 16S rRNA sequences using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). Next, these chosen regions were compared to all 16S rRNA sequences of target and target-related species. Based on sequence dissimilarities of over 10–15% compared to other species sequences, capture probe candidates were designed with similar melting temperatures using Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA). Finally, capture probe candidates were searched using the RDP II database and NCBI Blast searches to confirm their similarities with other bacteria. Consequently, new 12 specific capture probe candidates of five species were designed based on the above criteria (Supporting information, Table S2).

Next, the designed DNA oligonucleotide capture probe candidates were chemically synthesized with 5'-end modification (Integrated DNA Technology, IA, USA). After the experimental selection of the capture probe candidates (Supporting information, Fig. S1 and S2, Table S3), seven specific capture probes were ultimately selected from the newly designed candidates. A total of 27 specific capture probes including 20 previously designed capture probes [16] (Table 1), were used to discriminate 15 important pathogenic species. Positive control and artificial standard capture probes [18, 19, 25] were also employed (Table 1).

### 2.3 Design of the geno-biochip format

The DNA geno-biochip was prepared with a format in which each specific capture probe was surrounded by four rectangular-shaped spots of artificial standard capture probes (Fig. 1A). The specific capture probes were immobilized as four-repeated spots. The array used for the selection of capture probes contained  $34 \times 5$  spots of the artificial standard capture probe,  $1 \times 4$  spots of the positive control capture probe on the first line, and  $1 \times 4$  spots of each specific capture probe (to include a total of 32 probes) (Supporting information, Fig. S1). After probe selection, the final geno-biochip contained  $29 \times 5$  spots of the artificial standard capture probe,  $1 \times 4$  spots of the positive control capture probe on the first line, and  $1 \times 4$  spots of each specific capture probe (to include a total of 27 probes) (Fig. 1A).

### 2.4 Preparation of the geno-biochip

The geno-biochip was fabricated following previously described procedures [16, 18, 19]. Specifically,  $\text{NH}_2$ -mod-

**Table 1.** 16S rRNA–derived DNA oligonucleotide capture probes used in this study and their thermodynamic properties<sup>a)</sup>

Bacteria	Probe name	Sequences (5'-3', 5'-amine-spacer, spacer: C6)	Length (bp)	T <sub>m</sub> (°C)	Rating	Reference
All bacteria (positive control)	POCO	GCCGCCAGCGTTCAATCTGA	20	66.5	91	[16]
<i>Bacillus cereus</i>	BACE-1	CCTCGCGGTCTTGAGCTCTT	20	63	81	this study
	BACE-2	CCACCTGTCACTCTGCTCCCG	21	65	100	this study
<i>Campylobacter jejuni</i>	CAJE-1	CACTCTAGACTATCAGTTTCCCAAGC	26	68.6	88	this study
	CAJE-2	TACCCCTACACCACCAATTCATCTG	26	67.3	91	this study
<i>Clostridium perfringens</i>	CLPE	CGGAGGTGTTGAAACCCCCA	20	65	100	this study
<i>Escherichia coli</i>	ESCO	GAAGGCACATTCTCATCTCTGAAAAC	26	62.9	91	[16]
<i>Escherichia coli</i> O157:H7	ESCOO-1	CAGCAAAGAAGCAAGCTTCTTCCT	24	63.8	73	[16]
	ESCOO-2	ACTCGTCAGCAAAGAAGCAAGCT	23	62.7	86	[16]
<i>Listeria monocytogenes</i>	LIMO-1	GCATGCGCCACACTTTATCATT	22	62.6	80	[16]
	LIMO-2	CCATCTTTCAAAGCGTGGCAT	22	64.0	90	[16]
<i>Salmonella enterica</i> subsp. <i>enterica</i> serotype Cholerasuis	SACH-1	TGCTGCGGTTATTAACCAACAACA	23	63.2	86	[16]
	SACH-2	GACTCAAGCCTGCCAGTTTCGA	22	64.2	87	[16]
<i>Salmonella enterica</i> subsp. <i>enterica</i> serotype Enteritidis	SAEN	AGGCACAAATCCATCTCTGGATTC	24	63.5	74	[16]
<i>Shigella boydii</i>	SHBO-1	CCCCACCAACAAGCTAATCCC	21	63.1	57.1	this study
	SHBO-2	ACATTCTCATCTCTGAAAACCTCCGT	26	62.1	92	this study
<i>Shigella dysenteriae</i>	SHDY-1	AGGCACCCTCGTATCTCTACAAGG	24	63.1	89	[16]
	SHDY-2	CCGCCACTCGTCAGCAAAGCA	21	68.9	100	[14]
<i>Staphylococcus aureus</i>	STAU-1	AACTAGCTAATGCAGCGCGGAT	22	63.4	81	[16]
	STAU-2	AGATGTGCACAGTTACTTACACATATGTTCT	31	63.0	74	[16]
<i>Vibrio cholera</i>	VICH-1	CCTCTACCGGGCAATTTCC	19	63.5	79	[16]
	VICH-2	CTCTACCGGGCAATTTCCA	20	62.3	79	[16]
<i>Vibrio parahaemolyticus</i>	VIPA-1	CCCGAAGGTTTCAGATAACTCGTTT	24	63.1	88	[16]
	VIPA-2	CGTTATCGTTCCCCGAAGTTCAGAT	25	66.9	93	[14]
<i>Vibrio vulnificus</i>	VIVU-1	AAACAAGTTTCTCTGTGCTGCCGC	24	66.9	91	[14]
	VIVU-2	TGAGCCGAAGCTATCATGCCG	21	65.8	83	[14]
<i>Yersinia enterocolitica</i>	YEEN-1	GTTATTGGCCTTCTCTCGCT	22	63.8	84	[16]
	YEEN-2	TGCCAGTAACGTCAATCCAACAA	23	63.1	89	[16]
Artificial standard	ARST	CCCAAGGGAACCAAGGGAAA	21	66.8	85	[23]
Artificial standard target	ARSTT	TTTCCTTGGGTTCCTTGGG-Alexa flour 647	21	66.8	85	[23]

a) If thermodynamic properties were calculated by Primer Premier.

ified DNA oligonucleotide probes were spotted on aldehyde-coated slides (Super Aldehyde; Telechem International, Sunnyvale, CA, USA). Each DNA oligonucleotide (10 µM) was dissolved in a 3x SSC spotting buffer solution (450 mM NaCl, 3 mM tri-sodium citrate, and *N,N,N*-trimethyl glycine (betaine; Sigma); pH 6.6, final concentration 1.5 M). The oligonucleotide capture probes were printed on the slides using a Microsys 5100 microarrayer (Cartesian Technologies, Ann Arbor, MI, USA) with the Chip Maker 2 pin (Telechem International) at 74% humidity in a class 10 000 clean room. After spotting and overnight incubation in low (~30%) humidity conditions, the genobiochip slides were dipped in a solution containing 1.3 g of NaBH<sub>4</sub> dissolved in 375 mL of phosphate-buffered saline (PBS; pH 7.4) and 125 mL of ethanol for 5 min, fol-

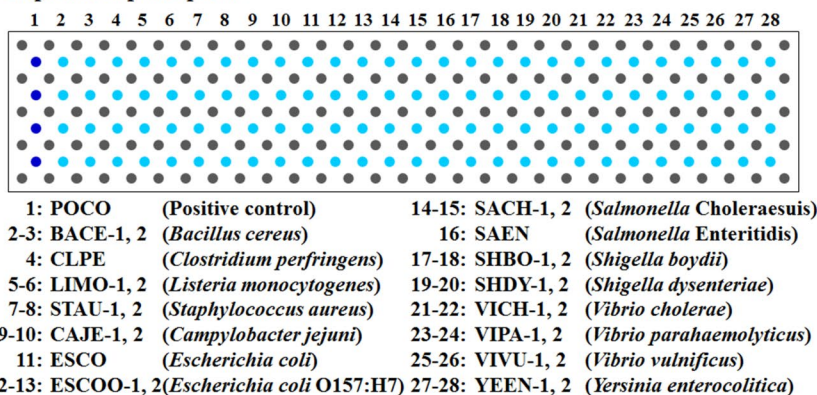
lowed by washing twice in 0.2% sodium dodecyl sulfate (SDS) for 1 min each, and twice with distilled water (DW). Slides were dried by centrifugation at 1500 rpm for 3 min, and stored at room temperature under a vacuum until further use.

## 2.5 Genomic DNA isolation and 16S rDNA target preparation

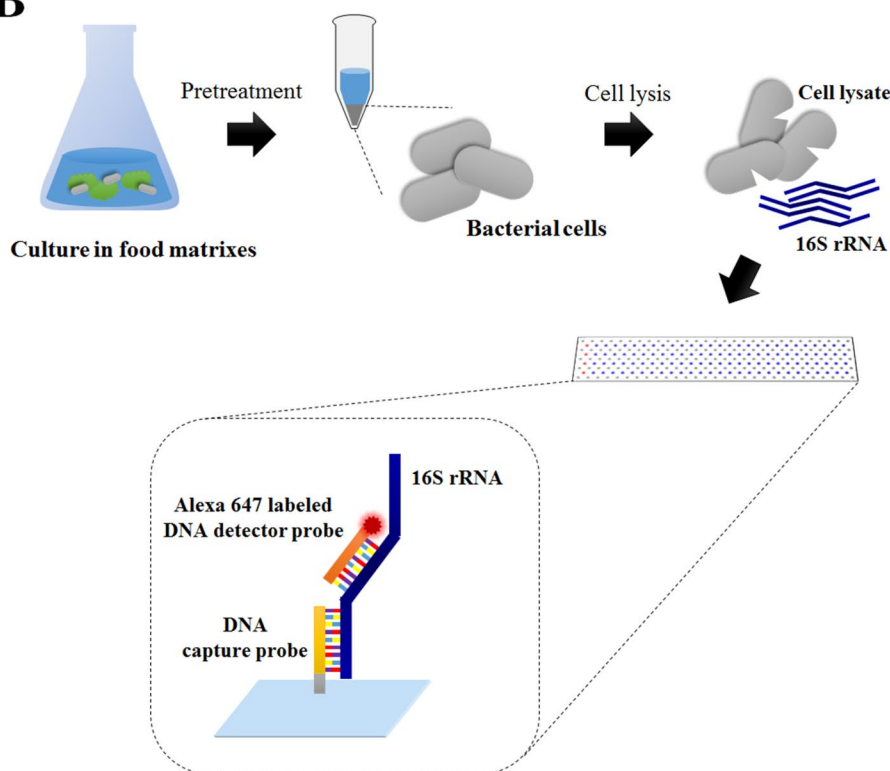
All species were cultured in nutrient broth (Difco, Kansas, MO, USA) at 30 or 37°C, except for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, which were cultured in trypticase soy broth (TSB; Difco) with 2% NaCl at 37°C and *C. perfringens*, which was cultured in reinforced clostridial medium (Difco) at anaerobic 37°C. The strains

A

- Artificial standard probe
- Positive control probe
- Specific capture probe



B



**Figure 1.** Schematic diagrams of (A) the array format for the 16S rRNA-derived geno-biochip containing 27 specific capture probes and (B) the schematic steps of the 16S rRNA detection of bacterial cells directly separated from food matrices.

of *E. coli* O157:H7 and *C. jejuni* were acquired as purified genomic DNAs. Each genomic DNA was extracted and purified using DNeasy® Tissue Kit (Qiagen GmbH, Hilden, Germany). Genomic DNA concentration and purity were checked with a UV/vis spectrometer (Shimadzu, Kyoto, Japan).

Each purified genomic DNA was used as a template for the amplification of 16S rDNA using PCR, followed by fluorescence labeling of each DNA target. The universal primer set (forward: 5'-AGAGTTTGATCMTGGCTCAG-3',

backward: 5'-TACGGYTACCTTGTACGACTT-3'; Genotech, Daejeon, Korea) used in this study includes the first and ninth conserved regions of 16S rDNA [22]. The PCR mixture was composed of 1–50 ng/mL of genomic DNA, 2 U *Taq* polymerase (Takara, Otsu, Japan), 2 μM forward universal primer, 2 μM reverse universal primer, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP, 0.15 mM amine-modified dUTP, and 1x *Taq* buffer. PCR was performed in the DNA thermal cycler (BioRad, Hercules, CA, USA) under the following conditions: 95°C for

5 min, 30 cycles of 95°C for 1 min, 60°C for 45 s, and 72°C for 1 min 30 sec, followed by 72°C for 5 min. A Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA) was employed for the purification of amine-modified amplicons, followed by ethanol precipitation. Next, each purified 16S rDNA amplicon was labeled using an ARES™ Alexa Fluor® 647 DNA labeling kit (Molecular Probes, Eugene, OR, USA) and purified by the PCR clean-up kit once more before use.

## 2.6 Hybridization and fluorescence intensity scanning

Before hybridization, the fabricated geno-biochip was pre-incubated in the buffer containing a 3x SSC solution (450 mM NaCl and 3 mM tri-sodium citrate; pH 7.0) with 1% w/v bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and 0.1% w/v SDS for 30 min at 50°C. The array was washed three times (twice with DW and once with ethanol) and dried by centrifugation at 1500 rpm for 3 min. Then, the array was spiked with the hybridization solution (3x SSC, 0.1% w/v SDS, and 0.2% w/v BSA) at 50°C for 1 h containing 50–150 µg/mL PCR-amplified target 16S rDNA and 1 µM artificial standard target DNA [25]. Next, the array was washed four times: the first with buffer I (1x SSC and 0.2% w/v SDS) for 1 min, the second with buffer II (0.1x SSC and 0.2% w/v SDS) for 1 min, and the third and the fourth with buffer III (0.1x SSC) for 1 min at room temperature. After drying, the geno-biochip was scanned using a commercial confocal laser scanner (ScanArray Lite; GSI Lumonics, Wilmington, MA, USA), and the data were analyzed using quantitative microarray analysis software (QuantArray; GSI Lumonics).

## 2.7 Direct RNA-detection of pathogen with eight food matrixes

A total of eight foods were tested as the model for each food matrix: rice for the grain matrix, pork for the meat matrix, eggs for the livestock matrix, canned corn for the processed agricultural matrix, fish cakes for the processed seafood matrix, milk and cheese for the processed livestock matrix, and ham for the processed meat matrix. As a model target pathogen, *S. Enteritidis* was cultured in nutrient broth with 12.5% w/v of each food at 37°C for one day. To separate the bacterial cells from food matrixes, each food matrix was pretreated by different procedures. The samples of rice, canned corn, and fish cakes were centrifuged at 500 rpm for 1 min to remove the large volume of solids. Then, each supernatant was collected and recentrifuged at 13 000 rpm for 1 min. The pellet was washed three times using PBS solution. The cheese sample was centrifuged at 13 000 rpm for 1 min to remove the floating matters and the rest of the process was same as previously described. The egg sample was centrifuged at 13 000 rpm for 2 min. Layers of bacterial cells and egg-food

solids were formed, and the layer of bacterial cells was resuspended into the supernatant. After centrifugation at 13 000 rpm for 1 min, the cell pellet was washed three times with PBS. The milk sample, which was a liquid, did not require removal of particles. The meat sample, which could not be separated with bacterial cells, was centrifuged at 13 000 rpm for 1 min. The cell pellet was washed as previously described.

After the pretreatments of food matrixes, each cell pellet was stored in a deep freezer (−80°C) for at least 30 min and was then resuspended in lysis buffer (1 M NaOH, 0.1% Triton X-100, and 2 mM EDTA in 20 mM Tris-HCl pH 8.0) with 1 mg mL<sup>−1</sup> lysozyme for 5 min at room temperature. After incubation, 1 M sodium phosphate buffer (pH 7.2) was added and mixed. Immediately, the prepared cell lysate containing the total RNAs was mixed with the hybridization buffer (4x SSPE (0.6 M NaCl, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, and 4 mM EDTA), 0.4x Denhardt's solution, 30% v/v formamide, 0.2 µM Alexa 647-labeled artificial standard target [23], 0.2 µM Alexa 647-labeled detector probe (DP, Universal 2) [19] and hybridized onto the developed 16S rRNA-derived geno-biochip at room temperature for 1 h (Fig. 1B). After hybridization, the biochip was washed three times: first with buffer I (1x SSPE and 0.2% v/v Triton X-100) for 30 s, second with buffer II (0.1x SSPE and 0.2% v/v Triton X-100) for 30 s, and third with buffer III (0.1x SSPE) for 30 s at room temperature. After drying, the biochip was scanned using a commercial confocal laser scanner, and the data were analyzed using quantitative microarray analysis.

## 3 Results

### 3.1 Design and selection of 16S rRNA-derived DNA oligonucleotide specific capture probes

Previously, 20 specific capture probes were designed and selected for 10 pathogenic bacteria [18]. To design capture probes for the specific detection of five new pathogens (*B. cereus*, *C. jejuni*, *C. perfringens*, *S. boydii*, and *S. sonnei*), all collected 16S rRNA sequences of target bacteria were aligned and analyzed to obtain DNA sequence fragments 20–25 bp in length with over 10–15% dissimilarity. The matching analysis based on the RDP II database and NCBI blast search was performed to confirm the dissimilarity of searched gene fragments between specific bacteria and others. After searching the sequence fragments, their thermodynamic properties were analyzed to select 12 capture probe candidates, which have similar melting temperatures, with formerly designed probes with differences within 2°C (Supporting information, Table S2).

Before the hybridization experiments, an *in silico* analysis was performed based on sequence comparison between the directly sequenced data and the databases (data not shown). Most sequences of capture probe can-



	POCO	BACE-1	BACE-2	CLPE	LIMO-1	LIMO-2	STAU-1	STAU-2	CAJE-1	CAJE-2	ESCO	ESCOO-1	ESCOO-2	SACH-1	SACH-2	SAEN	SHBO-1	SHBO-2	SHDY-1	SHDY-2	VICH-1	VICH-2	VIPA-1	VIPA-2	VIVU-1	VIVU-2	YEEN-1	YEEN-2
<i>B. cereus</i>	35.4	7.4	1.9	1.3	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1
<i>C. perfringens</i>	21.0	1.1	1.0	10.6	1.1	0.9	1.0	1.1	1.1	1.1	1.0	1.0	1.0	0.9	0.9	1.0	1.1	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.1	1.1
<i>L. monocytogenes</i>	23.1	1.1	1.1	1.0	2.7	5.2	2.9	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
<i>S. aureus</i>	23.4	1.1	1.0	1.1	1.0	1.0	1.0	55.5	1.4	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.3	1.0	1.0	1.0	1.0
<i>C. jejuni</i>	10.3	1.0	1.0	0.9	0.9	0.9	1.0	1.0	6.0	3.7	1.1	1.0	1.1	0.9	1.0	1.0	1.0	1.1	1.0	2.8	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.1
<i>E. coli</i>	57.9	1.6	1.0	1.0	1.0	1.0	1.1	1.0	1.1	19.6	1.9	12.9	1.0	1.1	1.2	8.8	25.6	1.1	54.6	1.2	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0
<i>E. coli</i> O157:H7	19.8	1.0	1.0	0.9	1.0	0.9	1.0	1.0	1.0	0.9	1.9	9.9	1.0	1.0	1.0	0.9	1.0	1.0	21.6	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0
<i>S. Choleraesuis</i>	60.8	3.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	11.9	1.1	7.6	22.1	55.8	2.0	6.3	11.7	1.2	63.6	1.2	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0
<i>S. Enteritidis</i>	50.5	1.5	1.1	1.0	1.2	1.0	1.1	1.0	1.0	1.0	1.0	2.2	9.1	40.8	16.1	2.8	1.0	1.1	42.3	1.1	1.0	1.0	1.1	1.1	0.9	1.1	1.1	1.1
<i>S. boydii</i>	43.2	1.8	1.0	1.0	0.9	0.9	1.0	1.0	0.9	1.0	36.9	1.3	1.1	1.0	1.1	1.1	41.5	45.3	1.3	43.8	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.0
<i>S. dysenteriae</i>	25.0	2.1	1.2	1.0	1.0	0.9	0.9	1.0	1.0	1.0	0.9	1.0	7.6	0.9	0.9	0.9	0.9	0.8	23.2	23.9	0.9	0.8	0.8	1.0	1.0	0.8	0.8	0.8
<i>V. cholerae</i>	31.9	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.0	0.9	0.9	1.0	1.0	1.0	1.1	1.0	0.9	0.9	1.0	1.0	3.2	3.7	0.9	0.9	1.5	1.0	0.9	0.9
<i>V. parahaemolyticus</i>	43.5	2.1	1.0	1.0	1.0	0.9	1.0	1.0	1.1	1.1	1.1	1.0	1.1	1.0	1.1	1.0	1.3	1.0	1.0	1.0	1.0	1.0	14.5	20.8	1.0	1.0	1.0	1.0
<i>V. vulnificus</i>	61.8	2.1	1.0	1.0	1.0	0.9	1.0	0.9	1.0	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.2	1.1	1.3	1.0	1.4	1.0	1.0	13.4	1.1	1.0	1.1	1.1
<i>Y. enterocolitica</i>	13.6	1.1	0.9	0.9	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.1	1.1	1.0	1.0	1.0	1.6	0.9	1.0	1.0	1.0	1.0	1.0	1.0	5.1	3.4
<i>S. sonnei</i>	1.7	1.0	1.0	1.0	0.9	1.0	1.0	0.7	1.0	1.0	1.9	1.0	1.0	1.0	1.0	1.0	2.4	1.8	1.1	6.6	1.1	1.0	1.1	1.0	1.0	1.0	1.4	1.1

**Figure 2.** The heat map plot of  $S/N$  of fluorescence intensities acquired from hybridization with each amplified target. Inner numbers indicate the  $S/N$  of each spots. The signals were marked as different colors: black for true-positive spots; gray for false-positive spots; and white for negative spots. Positive signals were determined when  $S/N$  was equal to or higher than 2, and negative signals were determined when  $S/N$  was lower than 2.

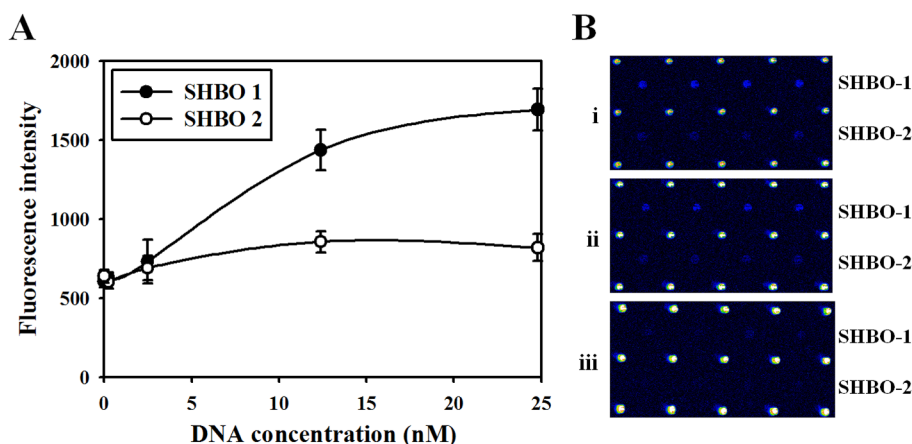
didates were the same as those of the respective bacterial genome database. Although some had mutations, these differences were presumed to not significantly affect the hybridization results because their dissimilarities were lower than 10% as the lowest threshold of the specificity. However, SHSO-1 (the capture probe for *S. sonnei*) showed low dissimilarity (9.5%) with *S. boydii*, indicating that the discrimination of *S. boydii* with *S. sonnei* might be difficult based on the fluorescence signal of SHSO-1 due to the nonspecific false-positive signal.

The detection abilities of newly designed 12 capture probe candidates were examined using hybridization experiments with 20 previously designed capture probes (Table 1). For the experiments, the probe screening geno-biochip was fabricated to contain a total of 33 capture probes (Supporting information, Fig. S1). 16S rDNA PCR amplicons of six bacteria including *B. cereus*, *C. jejuni*, *C. perfringens*, *S. boydii*, *S. sonnei*, and *S. dysenteriae* were tested to confirm the selectivity and cross-reactivity of 12 capture probe candidates (Supporting information, Fig. S2). Most of the newly designed specific capture probes showed true-positive signals with their target bacteria, but three spots of CLPE-2, SHBO-3, and SHSO-2 were false-negative even though target DNAs were hybridized (Supporting information, Figs. S2B–S2F, Table S3). Unfortunately, BACE-3 spots were frequently observed as false-positive signals for every tested pathogenic bacterium (Supporting information,

Figs. S2B–F, Table S3). Three *Shigella* spp., including *S. dysenteriae*, were tested to determine the cross-reactivity among them, and they displayed several false-positive signals with specific probes of other *Shigella* spp. or *E. coli* (Supporting information, Figs. S2D–F, Table S3). Based on repeated experiments, the three capture probes of false-negatives (CLPE-2 and SHBO-3) and false-positive (BACE-3) were excluded. In addition, two capture probes (SHSO-1 and SHSO-2) of *S. sonnei* were excluded because they were strongly false-positive with other target bacteria and ineffective for detection.

### 3.2 Multiple detection of 16 pathogens using amplified target DNA

After the selection of seven specific capture probes for five new pathogens, the complete 16S rRNA-derived geno-biochip containing 27 capture probes, including 20 previously designed capture probes was fabricated for the multiple detection of a total of 16 pathogenic bacteria (Fig. 1A). Although the specific capture probes of *S. sonnei* were excluded for final construction of the geno-biochip, we also conducted its detection because it was expected that *S. sonnei* would be discriminated by different signal pattern from *Shigella* spp. The detected spots of hybridization images (Supporting information, Fig. S3) were represented by a heat map plot of signal to noise ( $S/N$ ) to comprehend detection signals (Fig. 2). Positive



**Figure 3.** Sensitivity of the 16S rRNA-derived geno-biochip. The diluted PCR amplicons of *S. sonnei* were used for sensitivity determination. **(A)** The plot of dynamic detection ranges based on the fluorescence intensities according to target DNA concentration changes for each capture probe SHBO-1 (closed circle) and SHBO-2 (open circle). **(B)** Raw hybridization images for (i) 24.8 nM, (ii) 12.4 nM, and (iii) 2.48 nM 16S rDNA targets. Each value of plot was the mean of four repeated spots, and the error bars represent standard deviation.

spots were determined if  $S/N$  was equal to or higher than 2, and negative spots were determined if  $S/N$  was lower than 2 [26].

As a result, 16 target bacteria were successfully discriminated by their specific patterns (Fig. 2). However, similar to previous studies [16, 18], strains of *Shigella*, *Salmonella*, and *E. coli* had false-positive signals with mutual capture probes. Among the newly designed capture probes, SHBO-1 and SHBO-2 showed false-positive signals with target amplicons of *E. coli* and *S. Choleraesuis*. Although there were some false-positive spots, the developed 16S rRNA-derived geno-biochip containing 27 specific capture probes was able to successfully detect 16 target pathogens based on their signature signal patterns.

### 3.3 Sensitivity of the developed 16S rRNA derived geno-biochip

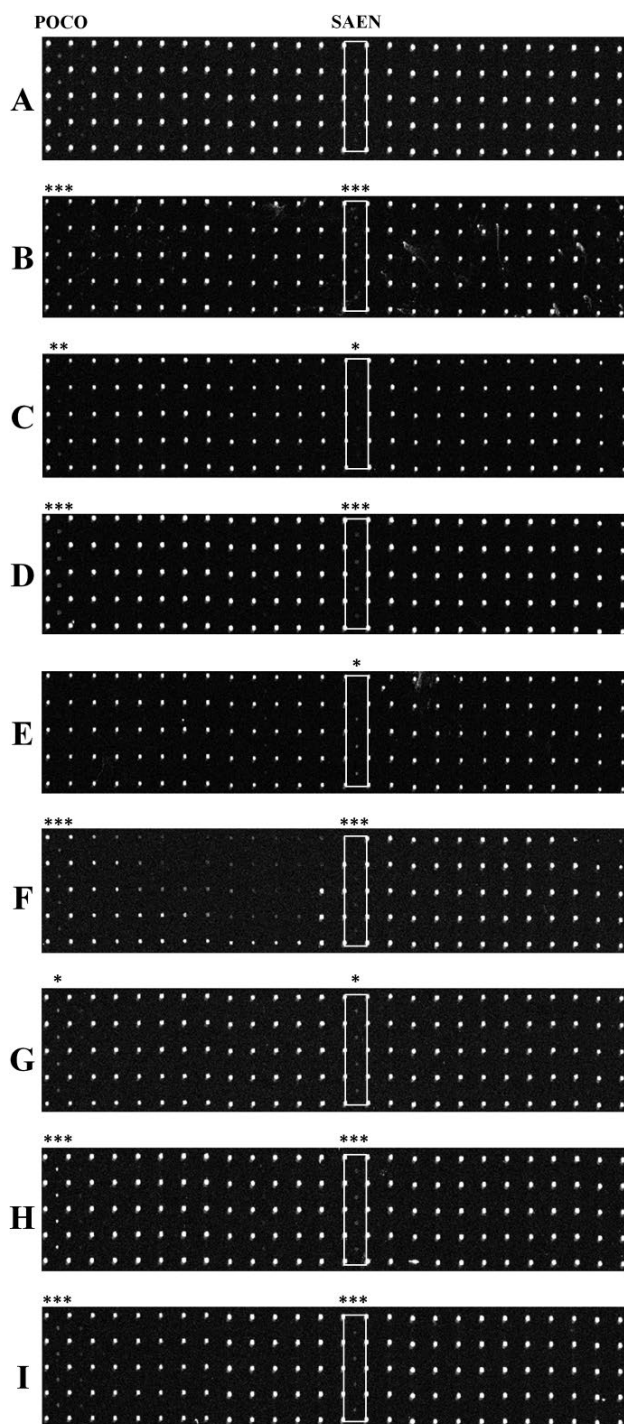
The sensitivity of the developed 16S rRNA-derived geno-biochip was measured using *S. boydii* (Fig. 3) as a representative strain. The five diluted 16S rDNA amplicons (0–24.8 nM) of *S. boydii* were prepared and applied to the hybridization tests. Then, the dynamic detection ranges were plotted based on the fluorescence intensities according to the concentration changes of target DNA. The fluorescence intensities of both SHBO-1 and SHBO-2 were linear below the concentration of 12.4 nM and saturated above that concentration. Both SHBO-1 and SHBO-2 signals were not observed at the point of 0.25 nM and the fluorescence intensity was lower or similar with that at the point of 0 nM. Thus, the limit of detection (LOD) was estimated as a range of 0.25–2.5 nM, which was calculated to be 10–100 fmol of the PCR product.

### 3.4 Direct RNA-detection of pathogen using target RNAs followed by the pretreatment of eight food matrixes

To apply the 16S rRNA-derived geno-biochip system for food inspection, it was necessary to develop the pretreat-

ment method for the isolation of bacteria from various complex food matrixes. To test these food matrixes, eight foods were chosen as representative models for each food matrix and bacteria were cultured in medium with each food: rice for a grain matrix, pork for a meat matrix, eggs for a livestock matrix, canned corn for a processed agricultural matrix, fish cakes for a processed seafood matrix, milk and cheese for a processed livestock matrix, and ham for a processed meat matrix. The pretreatment of food matrixes was simply accomplished using centrifugation. In the case of food matrixes with relatively heavy solids (e.g., rice, canned corn, and fish cakes), the bacterial cells were separated from foods using low-rpm centrifugation. Because milk is a liquid, it did not require removal of the solids and its pretreatment was the simplest. The egg and cheese samples, which retained tiny floating particles, required centrifugation at 13 000 rpm. The bacterial separation was relatively more difficult than for the solid matrixes. In the pork samples, it was impossible to remove the meat particles from bacterial cells just using centrifugation; thus, it was used without pretreatment. As a result, bacterial cells could be obtained from various food matrixes by simple pretreatment using centrifugation.

After the pretreatment of food matrixes, RNAs containing 16S rRNAs directly obtained from bacterial cells were applied to the geno-biochip detection system. As the representative target pathogen, *S. Enteritidis* acquired from each food matrix showed specific spots in the hybridization results (Fig. 4). The hybridization results of the pork sample showed the high fluorescence intensity of the background. The fluorescence intensities of specific spots for the egg and cheese samples were also lower than for those of other food samples. Despite these relatively low fluorescence intensities, it was confirmed that pathogens from contaminated food matrixes could be directly detected using our 16S rRNA-derived geno-biochip detection system.



**Figure 4.** The raw images of hybridization with complete RNA targets directly obtained from isolated *S. Enteritidis* cells in food matrixes using the rRNA-derived geno-biochip in Fig. 1. (A) control, (B) pork, (C) egg, (D) milk, (E) rice, (F) cheese, (G) canned corn, (H) ham, and (I) fish cake. White box indicates specific spots. Asterisks on images represent each *p*-value range of POCO and SAEN calculated by paired *t*-tests. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , and \*\*\* indicates  $p < 0.0005$ .

## 4 Discussion

In the present work, the multiple detection of 16 target pathogens based on signature signal patterns was successful using our developed 16S rRNA-derived geno-biochip containing 27 elaborately designed specific capture probes. The selection of capture probes was based on optimal probe design principles and validation steps of (i) the sequence dissimilarity over 10–15%, (ii) thermodynamic properties, (iii) database matching analysis, (iv) in silico analysis, and (v) probe screening experiments. As a result, capture probes were selected to maximize true-positive signals and minimize false-positive signals. Although some false-positive signals were inevitable due to the high sequence similarity of 16S rRNAs, each bacterium was identified by the characteristic pattern in the rows shown in Fig. 2.

Unexpectedly, some capture probe candidates (CLPE-2, SHBO-3, and SHSO-2) elicited true-negative signals with their target bacteria. This may have been because the amplified PCR products share the small-fraction sequences because CLPE-2 is located near to the end of the amplified 16S rRNA gene. In addition, several factors, such as the secondary structure of PCR amplicons and fragmentation during denaturing steps, might hinder the hybridization between the capture probe and the target DNA [27–30].

The detection results of three *Shigella* spp. showed several false-positive signals by the capture probes related to *Salmonella*, other *Shigella* spp. or *E. coli*. These results coincide with the previous study [18]. It is well known that *Shigella* spp. are considered to be anaerobic biotypes of *E. coli* and highly-closed strains [31–33]. Thus, it was quite difficult to strictly discriminate only true-positive signals of specific probes and eliminate the false-positive signals among them due to their very analogous 16S rRNA genes [18, 33, 34]. Nevertheless, each distinctive signal pattern could identify each strain of *Shigella* spp., *Salmonella* spp., and *E. coli*. For examples, detection results of *E. coli* were different in signals of ESCO, SHBO-1, and SHBO-2 compared to signal pattern of *E. coli* O157:H7 (Fig. 2). The signal pattern of *S. Choleraesuis* had differences in signals of ESCO and SHBO-2 compared to that of *S. Enteritidis*. Three *Shigella* spp. also had all different signal patterns in the probes of ESCO, ESCOO-1, and SHBO-1. Consequently, based on the signature pattern according to each bacterium, all pathogenic bacteria could be specifically discriminated with our developed 16S rRNA-derived geno-biochip. Indeed, it might need to employ novel biomarkers such as virulence factor genes for more specific detections of *Salmonella*, *Shigella*, and *E. coli*. Previously, we conducted the study for specific discrimination of three *Salmonella* serotypes with a new biomarker gene [35]. However, because the use of such biomarker gene is restricted to detection of a particular bacterium, the 16S rRNA-derived geno-biochip might be



more suitable for multiple detection of various bacterial species in food samples.

The LOD of the developed 16S rRNA-derived geno-biochip was estimated using representative *S. boydii* strain in a range of 0.25–2.5 nM, equivalent to 10–100 fmol of the PCR product. The LOD of 86–172 fmol which was evaluated using the amplified *C. jejuni* 16S rDNA (Supporting information, Fig. S4) was also consistent with the LOD using *S. boydii*. In the previous study, it was reported that the sensitivity of 16S rRNA-derived geno-biochip ranged from 6 fmol of the PCR product for *Mycoplasma bovis*, *Streptococcus agalactiae*, and *Streptococcus pyogenes* to 12 fmol for *Staphylococcus aureus* [36]. Although the sensitivity of this study was somewhat lower than the previous report using the same detection markers, the specific capture probes designed in this study could also detect targets as low as the fmol level.

Most of the specific signals were detected directly using the developed 16S rRNA-derived geno-biochip system with total RNAs containing 16S rRNAs from isolated bacterial cells from contaminated food matrixes. While the use of genomic DNAs as target materials needs a heat-denaturation step to effectively detect the fluorescence signal [37], the denaturation step was not necessary for RNAs. The detection results using 16S rRNAs showed lower fluorescence intensities than those using amplified 16S rDNAs. This was mainly caused by the inherent unstable characteristics of RNA compared to DNA in experimental environments [38, 39]. Therefore, it might be necessary to increase RNA stability during test by employing several strategies such as addition of RNase inhibitor, use of RNA stabilization solution, lowering hybridization temperature, and optimization of magnesium ion concentration which were reported to help stabilization of RNA [40–43].

The fluorescence intensities of specific signals for the egg and cheese samples were lower than those of other food samples. This might be the reason why the bacterial cells were not perfectly collected during pretreatment because the cells were located between the heavy and light layers of these samples. The hybridization result of the pork sample showed a high background intensity. The reason could be that the nonspecific binding of detector probes caused by the sticky components of meat were not separated during the preparation. Although complete RNAs directly obtained from food matrixes were used as target materials, the detection was successful because 16S rRNAs are abundant ( $10^4$ – $10^5$  per cell) in bacterial cells as actual target nucleic acids [44]. The interferences by food matrixes were also reduced because most food particles could be removed simply using centrifugation.

Among pathogenic bacteria, 16 strains used in this study are important targets in the food industry and public hygiene, because they are major and frequent causes of outbreaks of foodborne disease [2, 45–47]. Therefore, our geno-biochip detection system could be a powerful

solution to check for foodborne pathogen safety. Collectively, the geno-biochip based on 16S rRNA information is able to selectively and practically detect multiple common bacterial pathogens in the food industry and public hygiene and would be useful to monitor food safety.

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*The authors declare no financial or commercial conflict of interest.*

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#### Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Hyung Joon Cha, Noriho Kamiya and S. Vikineswary Sabaratnam, covers the most advanced biotech research from Asian Congress of Biotechnology 2015. This issue includes articles on drug delivery, enzyme engineering, cellular therapy, biosensors, etc. The 30Kc19 protein derived from the silkworm hemolymph consists of two domains, which are 30Kc19 $\alpha$  (blue) and 30Kc19 $\beta$  (red). The cover image shows that 30Kc19 $\alpha$  has multifunctional properties, which are cell penetration, protein stabilization, and cargo delivery. The Image is provided by Jina Ryu, Hyoju Kim, Hee Ho Park, Hong Jai Lee, Ju Hyun Park, Won Jong Rhee and Tai Hyun Park authors of "Protein-stabilizing and cell-penetrating properties of  $\alpha$ -helix domain of 30Kc19 protein" (<http://dx.doi.org/10.1002/biot.201600040>).

### Biotechnology Journal – list of articles published in the November 2016 issue.

#### Editorial

##### Asian Congress on Biotechnology 2015

Hyung Joon Cha, Noriho Kamiya and  
S. Vikineswary Sabaratnam

<http://dx.doi.org/10.1002/biot.201600650>

#### Commentary

##### Therapeutic effects of stem cells on ischemic stroke were confirmed in an improved photothrombotic mouse model

I-Ming Chu

<http://dx.doi.org/10.1002/biot.201600414>

#### Review

##### Solid-in-oil nanodispersions for transdermal drug delivery systems

Momoko Kitaoka, Rie Wakabayashi, Noriho Kamiya  
and Masahiro Goto

<http://dx.doi.org/10.1002/biot.201600081>

#### Review

##### Design of nanoscale enzyme complexes based on various scaffolding materials for biomass conversion and immobilization

Jeong Eun Hyeon, Sang Kyu Shin and Sung Ok Han

<http://dx.doi.org/10.1002/biot.201600039>

#### Research Article

##### Effect of human mesenchymal stem cell transplantation on cerebral ischemic volume-controlled photothrombotic mouse model

Yun-Kyong Choi, Enerelt Urnukhsaikhan, Hee-Hoon Yoon,  
Young-Kwon Seo and Jung-Keug Park

<http://dx.doi.org/10.1002/biot.201600057>

#### Research Article

##### Multiplex 16S rRNA-derived geno-biochip for detection of 16 bacterial pathogens from contaminated foods

Hwa Hui Shin, Byeong Hee Hwang and Hyung Joon Cha

<http://dx.doi.org/10.1002/biot.201600043>

#### Research Article

##### Fabrication of multilayered vascular tissues using microfluidic agarose hydrogel platforms

Keita Kinoshita, Masaki Iwase, Masumi Yamada,  
Yuya Yajima and Minoru Seki

<http://dx.doi.org/10.1002/biot.201600083>

#### Research Article

##### Enhanced production of 2,3-butanediol in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* through optimizing ratio of glucose/galactose

Eun-Ji Choi, Jin-Woo Kim, Soo-Jung Kim, Seung-Oh Seo,  
Stephan Lane, Yong-Cheol Park, Yong-Su Jin and Jin-Ho Seo

<http://dx.doi.org/10.1002/biot.201600042>

#### Research Article

##### Ex vivo culture of circulating tumor cells using magnetic force-based coculture on a fibroblast feeder layer

Shuhei Yamamoto, Kazunori Shimizu, Jiahui Fei, Hiroji  
Iwata, Mina Okochi, Hayao Nakanishi and Hiroyuki Honda

<http://dx.doi.org/10.1002/biot.201600084>

#### Research Article

##### Protein-stabilizing and cell-penetrating properties of $\alpha$ -helix domain of 30Kc19 protein

Jina Ryu, Hyoju Kim, Hee Ho Park, Hong Jai Lee,  
Ju Hyun Park, Won Jong Rhee and Tai Hyun Park

<http://dx.doi.org/10.1002/biot.201600040>

Research Article

**Enzymatically prepared redox-responsive hydrogels as potent matrices for hepatocellular carcinoma cell spheroid formation**

*Kousuke Moriyama, Shono Naito, Rie Wakabayashi,  
Masahiro Goto and Noriho Kamiya*

<http://dx.doi.org/10.1002/biot.201600087>

Research Article

**Theoretical calculations on the feasibility of microalgal biofuels: Utilization of marine resources could help realizing the potential of microalgae**

*Hanwool Park, Choul-Gyun Lee*

<http://dx.doi.org/10.1002/biot.201600041>